

The chemokine receptor antagonist AOP-RANTES reduces monocyte infiltration in experimental glomerulonephritis

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The chemokine receptor antagonist AOP-RANTES reduces monocyte infiltration in experimental glomerulonephritis.

Background. This study was designed to evaluate the role of the novel chemokine receptor antagonist amino-oxypentane RANTES (AOP-RANTES), which blocks the binding of macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , and RANTES to the chemokine receptor-5 (CCR-5) on the infiltration of monocytes in experimental glomerulonephritis.

Methods. Rats were treated twice daily with 12.5 μ g AOP-RANTES following an induction of anti-rat-thymocyte antibody-mediated glomerulonephritis. The white blood cell count, glomerular monocyte infiltration, chemokine expression, and collagen type IV deposition were assessed.

Results. The induction of glomerulonephritis increased glomerular monocyte/macrophage (M/M) infiltration at 24 hours and at 5 days was still higher than in controls. AOP-RANTES prevented glomerular M/M infiltration at 24 hours and at 5 days. This was paralleled by reduced glomerular collagen type IV deposition as a fibrotic marker in nephritic animals.

Conclusion. These data show that the CCR-5 chemokine receptor antagonist AOP-RANTES ameliorates M/M infiltration and improves glomerular pathology in experimental glomerulonephritis. The use of chemokine receptor antagonists may offer a new therapeutic option in inflammatory renal injuries.

Chemokines are small secreted proteins that stimulate the directional migration of leukocytes and mediate inflammation [1]. The important role of chemokines in renal diseases has recently been reviewed [2, 3]. They exert their effects through binding on chemokine receptors [4, 5], predominantly expressed on leukocytes. Chemokine receptors are member of the seven-membrane-spanning, G-protein-coupled receptor family. The mechanism of coupling chemokine receptor activation to the process

of chemotaxis is still not fully understood. Some receptors are restricted to certain cells, whereas others are more widely expressed. A few of the receptors bind to only one chemokine, but others interact with several chemokines [4, 5].

In animal models of glomerulonephritis, chemokine-neutralizing approaches have shown that antibodies to interleukin-8 (IL-8) or monocyte chemoattractant protein-1 (MCP-1) can reduce the infiltration of either polymorphonuclear granulocytes, monocytes, or lymphocytes [6–10]. However, these approaches lead to only a 40 to 50% impairment of leukocyte infiltration, suggesting that more than one single chemokine is responsible for leukocyte recruitment. In fact, studies in the animal model of anti-glomerular basement membrane nephritis showed that in the development of glomerulonephritis, an up-regulation of several chemokines appears [11]. In addition to MCP-1, particularly macrophage inflammatory protein-1 α (MIP-1 α), interferon produced protein-10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and regulated on activated normal T cell expressed and secreted (RANTES) were increased. The blockade of one or more chemokines would be desirable as a potential therapeutic approach. Because most chemokine receptors on leukocytes bind more than one chemokine, the blockade of chemokine receptors could be a more efficient option. The recent development of amino-oxypentane (AOP)-RANTES, which is a modified chemokine (a derivative of RANTES that was created by chemical modification of the amino terminus) and a potent antagonist of the chemokine receptor-5 (CCR-5) on monocytes [12, 13], allowed us to study the possible role of a receptor antagonist on inflammatory cell recruitment of glomerulonephritis.

In our experiments, AOP-RANTES reduced glomerular monocyte infiltration and ameliorated the development of pathologic features of glomerulonephritis. AOP-RANTES may thus offer a new therapeutic option in glomerulonephritis.

Key words: monocyte chemoattractant protein-1, amino-oxypentane-RANTES, chemokine receptor-5, inflammatory renal injury.

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METHODS

Induction of glomerulonephritis and experimental design

Immune-mediated mesangial cell injury was induced in male Wistar rats (180 to 200 g/body wt) by intravenous injection of an anti-rat-thymocyte antiserum (ATS). ATS was induced in rabbits by repeated immunization with thymocytes from Lewis rats as described earlier [14]. The following groups of animals were studied ($N = 5$ for all groups and each time point): (a) controls, that is, animals that remained untouched until nephrectomy; (b) nephritis, animals given 0.5 ml/100 g/body wt ATS intravenously + 0.3 ml of distilled water intravenously; and (c) animals with nephritis + AOP-RANTES, 0.5 ml/100 g/body wt ATS intravenously + 12.5 mg AOP-RANTES in 0.3 ml distilled water intravenously twice daily.

The experiments were carried out in three complete sets.

Isolation of glomeruli and total RNA for Northern blot hybridization

Glomeruli were isolated by a fractional sieving technique as described earlier [14]. Cellular RNA from glomeruli was isolated by the guanidinium isothiocyanate method [15]. For Northern blot analysis, 20 μ g of total RNA were size fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to nylon membranes (Zetabind; Cuno, Meriden, CT, USA). Equal loading of the lanes was evaluated by ethidium bromide staining of the 18 and 28 S RNA. The membranes were hybridized with a 577 bp MCP-1 cDNA fragment and a rat RANTES cDNA fragment previously labeled with cytidine triphosphate (CTP) (32 P) as described earlier [16, 17] in hybridization buffer [$5 \times$ SSPE, $2 \times$ Denhardt's 150 μ g/ml, sonicated and denatured salmon sperm DNA (Sigma-Aldrich Chemie, Germany), 0.1% sodium dodecyl sulfate (SDS), 5% dextran sulfate, and 50% formamide] for 18 hours at 42°C. The membranes were washed to high stringency in standard saline citrate (SSC)/SDS at 65°C as described earlier [15]. Autoradiography was performed for 2 to 72 hours at -80°C . Membranes were rehybridized with a 580 bp cDNA probe of human 18 S RNA to account for small loading and transfer variabilities. Exposed films were quantitated by using phosphoimager Bio-Rad-GS-363 (multianalyst software). Northern blot experiments were independently performed for three different complete sets of experiments with qualitatively similar results.

RT-PCR for macrophage inflammatory protein-1 α

For reverse transcription-polymerase chain reaction (RT-PCR), 1 μ g of total RNA, 4 μ l $5 \times$ first strand buffer, 1 μ l dNTPs (20 mM), 2 μ l dithiothreitol (0.1 M), 0.4 μ l poly(dT) primer (1 mg/ml; Pharmacia, Freiburg, Germany), 0.4 μ l RNasin (Promega, Madison, WI, USA),

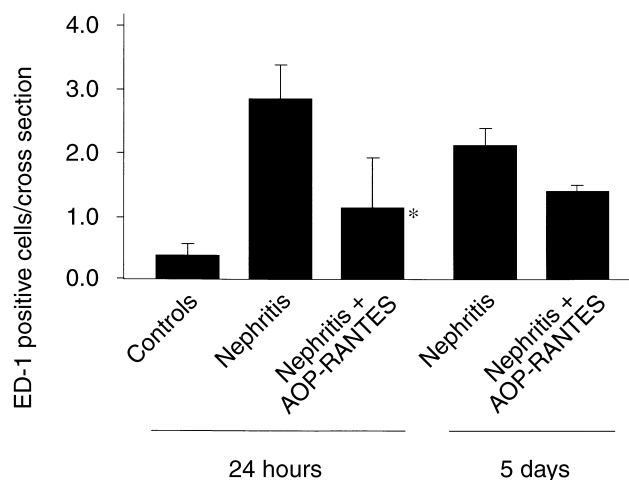


Fig. 1. Glomerular monocyte/macrophage (M/M) infiltration. The infiltration of M/M into the glomerulus was immunohistochemically assessed by staining with an antibody to ED-1. In nephritic animal, ED-1-positive cells increased significantly at 24 hours. AOP-RANTES reduced the glomerular cell number at both time points studied; however, the alterations were only significant at 24 hours versus nephritis ($*P = 0.02$). The cell numbers are expressed as ED-1-positive cells/glomerular cross-section. Data are given as means \pm SD.

and 1 μ l moloney-mouse leukemic virus (M-MLV) reverse transcriptase (2 U/ml; GIBCO BRL, Eggenstein, Germany) per sample was prepared. The RT reaction was carried out for two hours at 37°C. To perform PCR, the following sequence was used based on the following rat cDNA: forward primer, 5'CTT GCT GTT CTT CTC TGC ACC ATG 3', and reverse primer, 5'TCT CTT GGT CAG GAA AAT GAC ACC 3' (produced with Oligo 1000 AN Synthesizer; Beckmann, Munich, Germany). To 5 μ l of each sample, 29.5 μ l H₂O, 5 μ l $10 \times$ PCR-buffer, 3 μ l MgCl₂ (25 mM), 4 μ l dNTPs (2.5 mM), 1.5 μ l forward primer (50 ng/ml), 1.5 μ l reverse primer (50 ng/ml), and 0.5 μ l Taq DNA polymerase (5 U/ml; Promega) were added. PCR was run for 32 cycles using the following temperature profile: denaturation at 95°C for 10 seconds, annealing for 20 seconds at 57°C, extension for 30 seconds at 72°C. The length of the amplified fragment was 180 bp. RNA samples were also analyzed for the expression of the housekeeping gene GAPDH as described earlier [17]. Amplified cDNA fragments were separated on a 1.5% agarose gel, which contained 1 mg/ml ethidium bromide and was photographed on ultraviolet light using a Polaroid 655 film. Genomic DNA artifacts were ruled out by performing control PCR without RT. Exposed films were quantitated by using phosphoimager Bio-Rad-GS-363 (multianalyst software). RT-PCR experiments were independently performed for three different complete sets of experiments with qualitatively similar results.

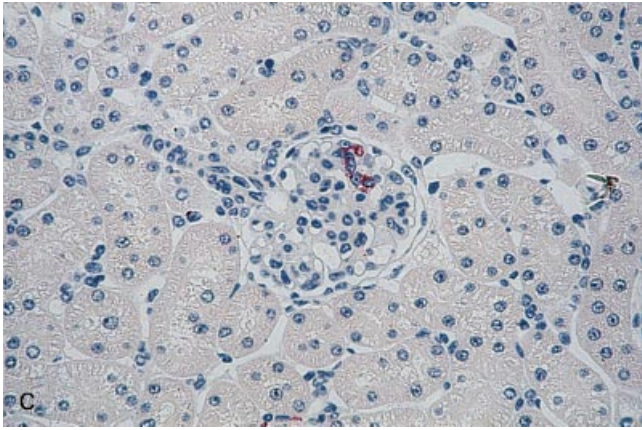
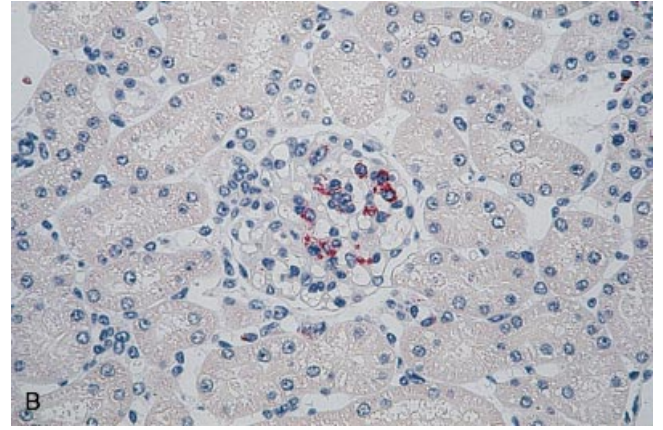
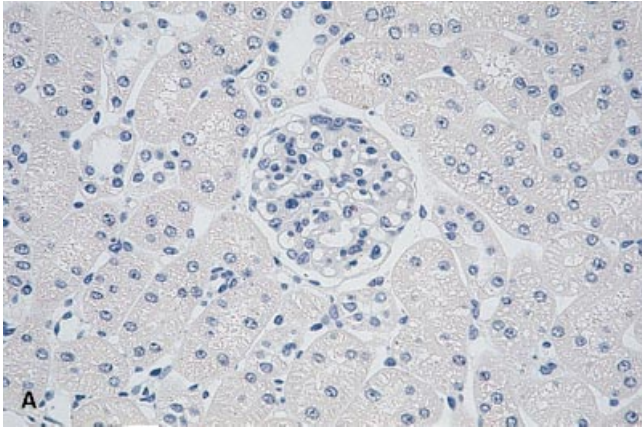


Fig. 2. Immunohistologic staining of glomerular monocyte/macrophage (M/M) infiltration. Immunohistologic staining of glomerular M/M infiltration with a ED-1 antibody revealed a marked increase of glomerular ED-1 positive cells 24 hours after induction of the anti-rat-thymocyte antiserum (ATS) nephritis (*B*) in comparison to the control group (*A*). When the nephritic animal received AOP-RANTES, the glomerular ED-1-positive cells were substantially reduced (24 hr; *C*; $\times 400$).

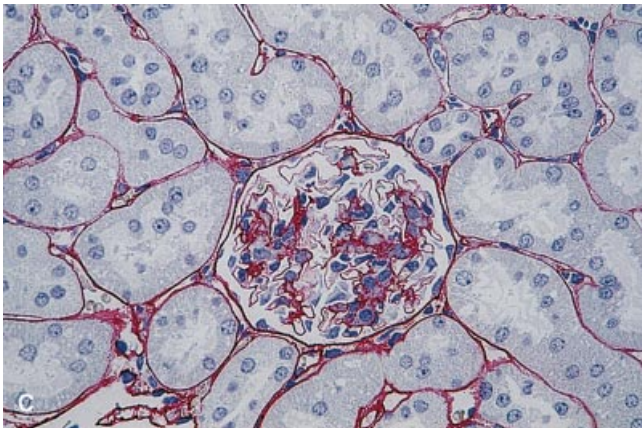
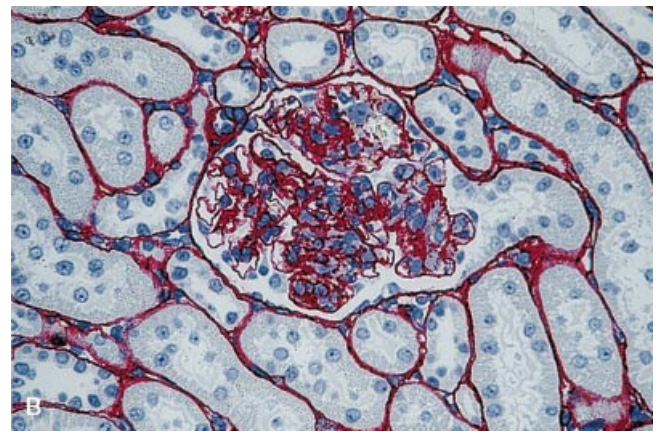
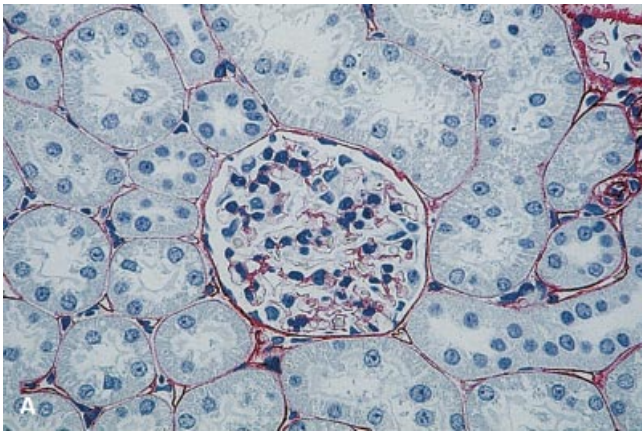


Fig. 4. Immunohistologic staining of glomerular collagen type IV deposition. Immunohistologic staining of renal tissue with a collagen type IV antibody demonstrated positivity in the glomerular mesangium and the capillary walls in control animals (*A*). Kidney sections of nephritic animals showed a increase of glomerular collagen type IV deposition five days after induction of the nephritis (*B*). The treatment of nephritic animals with AOP-RANTES leads to a significant reduction of glomerular collagen type IV deposition (*C*; $\times 400$).

Western blotting for RANTES and macrophage inflammatory protein-1 α

For Western blotting, glomeruli were isolated and centrifuged in $1 \times$ phosphate-buffered saline (PBS). The pellet was resuspended in 100 μ l of Laemmli buffer (33% vol/vol 0.5 mM Tris-HCl, pH 6.8, 66% vol/vol SDS 10%). Samples were boiled for 10 minutes and centrifuged. The protein concentration was determined with a modified Lowry method (Protein DC-assay; Bio-Rad, Richmond, CA, USA). Two equal amounts of protein (80 μ g), 1/4 vol % of Laemmli buffer 2 (50% β -mercaptoethanol, 50% glycerol) were added. The solution was loaded onto a polyacrylamide SDS-12% gel and was electrophoresed. A low molecular weight marker (Rainbow marker; Amersham, Braunschweig, Germany), which comprises 14.3 to 200 kDa, served as a molecular weight standard. After the completion of electrophoresis, proteins were electroblotted semidry (anode buffer I, 30 mM Tris, 20% methanol; anode buffer II, 300 mM Tris, 20% methanol; cathode buffer, 25 mM Tris, 40 mM 6-amionohexane acid, 20% methanol) for 45 minutes at 0.8 mA/cm² to a nitrocellulose membrane (Hybond ECL; Amersham). The membrane was blocked in 5% nonfat dry milk in washing buffer ($1 \times$ PBS, 0.1% Tween 20) for one hour at room temperature and was then incubated for another hour with a goat/anti-rat RANTES antibody or goat/anti-rat MCP-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) added to a concentration of 1:2000 in the same buffer. As size standards, recombinant RANTES (Pepro Tech, Inc., Rocky Hill, NJ, USA) and recombinant MCP-1 (Pepro Tech, Inc.) were used. After rinsing the membrane in washing buffer for 3×10 minutes, the secondary antibody, a rabbit antigoat immunoglobulin G conjugated to horseradish peroxidase (Southern Biotechnology, Birmingham, AL, USA) was added at a concentration of 1:1000. The luminescence detection of peroxidase was performed with ECL system according to the manufacturer's recommendations (Amersham) after rinsing membranes in washing buffer $1 \times$ PBS films were exposed 2 to 10 minutes at room temperature.

Histology

Kidney tissues were either fixed in 1% buffered formaldehyde or in Methyl Carnoy's solution. Tissue sections (2 μ m) were stained with the APAAP complex following an application of primary antibodies for immunohistology. Tissue was stained with the following antibodies: A goat antitype IV collagen antibody (Biozol, Hamburg Germany) and a monoclonal anti-ED-1 antibody (Chemicon International, Temecula, CA, USA). All quantitative morphologic analyses were performed in a blinded fashion. Evaluation of ED-1-positive cells was performed by counting positive cells in at least 80 glomeruli (range 80 to 110) of at least four kidneys at each time point as

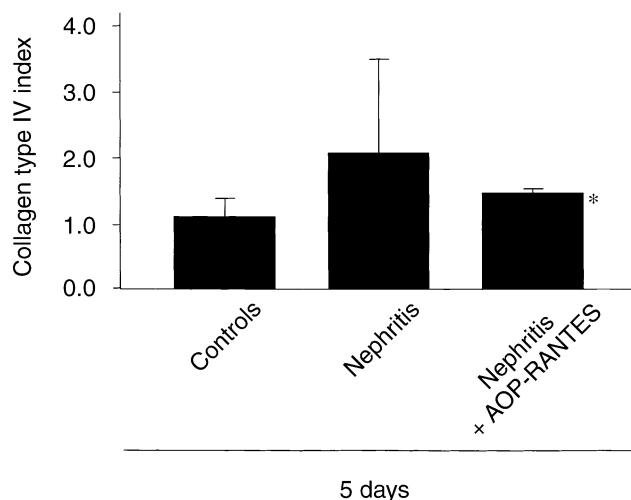


Fig. 3. Glomerular collagen type IV deposition. The quantitative assessment of glomerular collagen type IV deposition revealed that the *in vivo* treatment with AOP-RANTES significantly reduced glomerular collagen type IV deposition in nephritic animals at five days. * $P = 0.02$.

described earlier [18]. For the evaluation of extracellular collagen deposition, a modified score was used as described earlier [18]. At least 80 glomeruli were evaluated in each kidney section from at least four animals per time point. The percentage of glomerular matrix area stained by antitype IV collagen antibody was approximated and classified applying a score consisting of grades 0 through 4, grade 0 representing staining of 0 to 5% of the matrix area, grade 1 representing staining of 5 to 20%, grade 2 staining 20 to 40%, grade 3 staining of 40 to 60%, and grade 4 staining of more than 60%. Calculation of collagen deposition index was done in the following way: The number of glomeruli with a score 1 was multiplied by 1. Glomeruli with a score of 2 were multiplied by 2. Those with a score of 3 were multiplied by 3, and those with a score of 4 were multiplied by 4. These numbers were added and divided by the number of glomeruli assessed, including those with a score of 0.

For the immunohistochemistry of MCP-1, the kidneys were snap frozen in liquid nitrogen, and 8 mm thick cryostat sections were prepared and then fixed in acetone. The section were incubated with an anti-goat anti-rat MCP-1 antibody (Santa Cruz Biotechnology Inc.). After being incubated with biotin-conjugated second antibody, the tissue sections were processed using a streptavidin-biotin kit (ImmunoCruz Staining System; Santa Cruz Biotechnology). MCP-1 protein was visualized by incubation in peroxidase substrate, using diaminobenzidine as chromogen.

Statistical analysis

Results are expressed as means \pm SEM unless stated otherwise. Statistical significance was defined as $P < 0.05$.

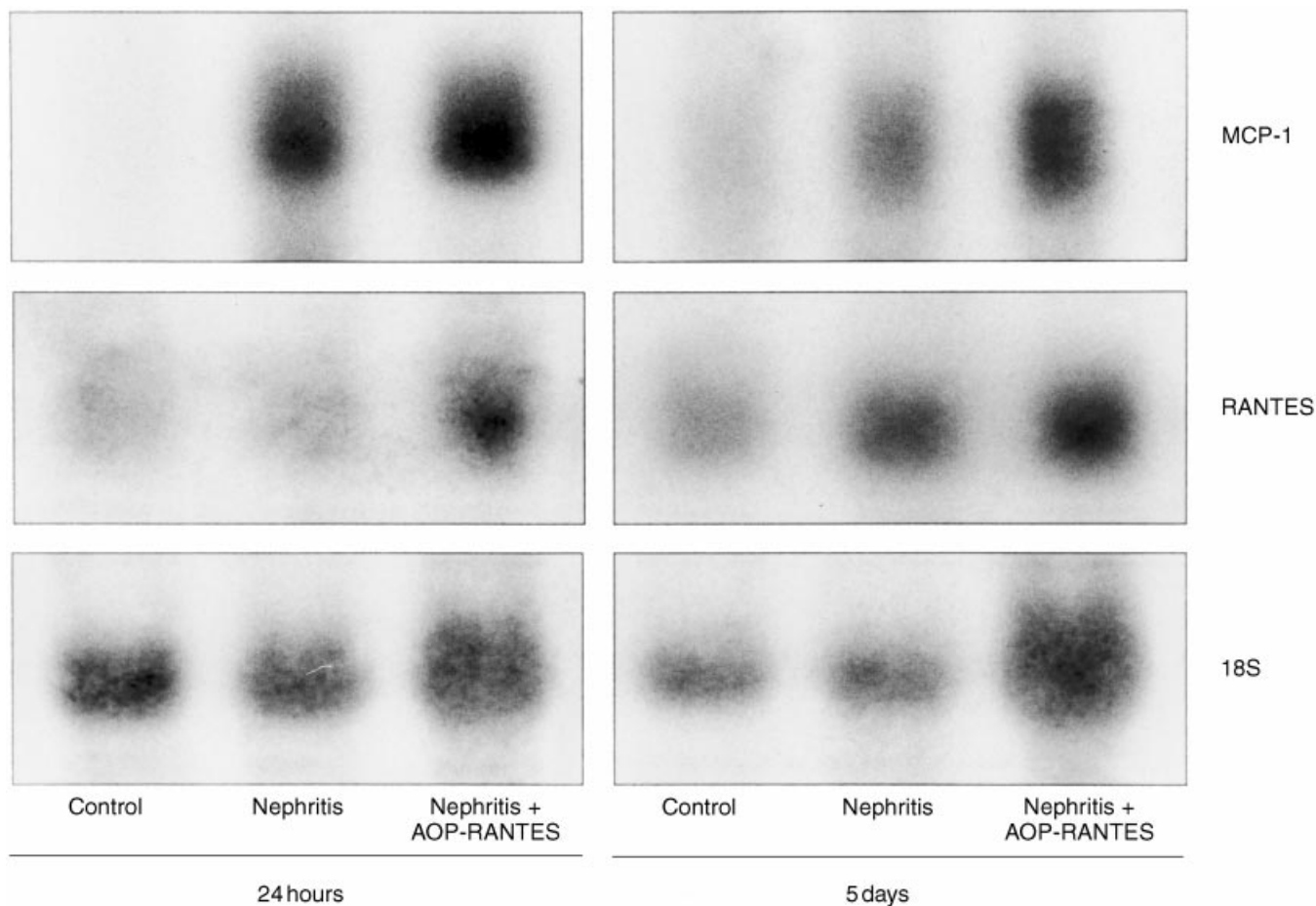


Fig. 5. Northern blot analysis. Glomerular mRNA levels of monocyte chemoattractant protein-1 (MCP-1) increased markedly at 24 hours and slightly at 5 days when compared with controls. RANTES mRNA expression was not increased 24 hours after nephritis induction but increased at 5 days. Animals treated with AOP-RANTES showed a similar RANTES and MCP-1 expression than nephritic animals.

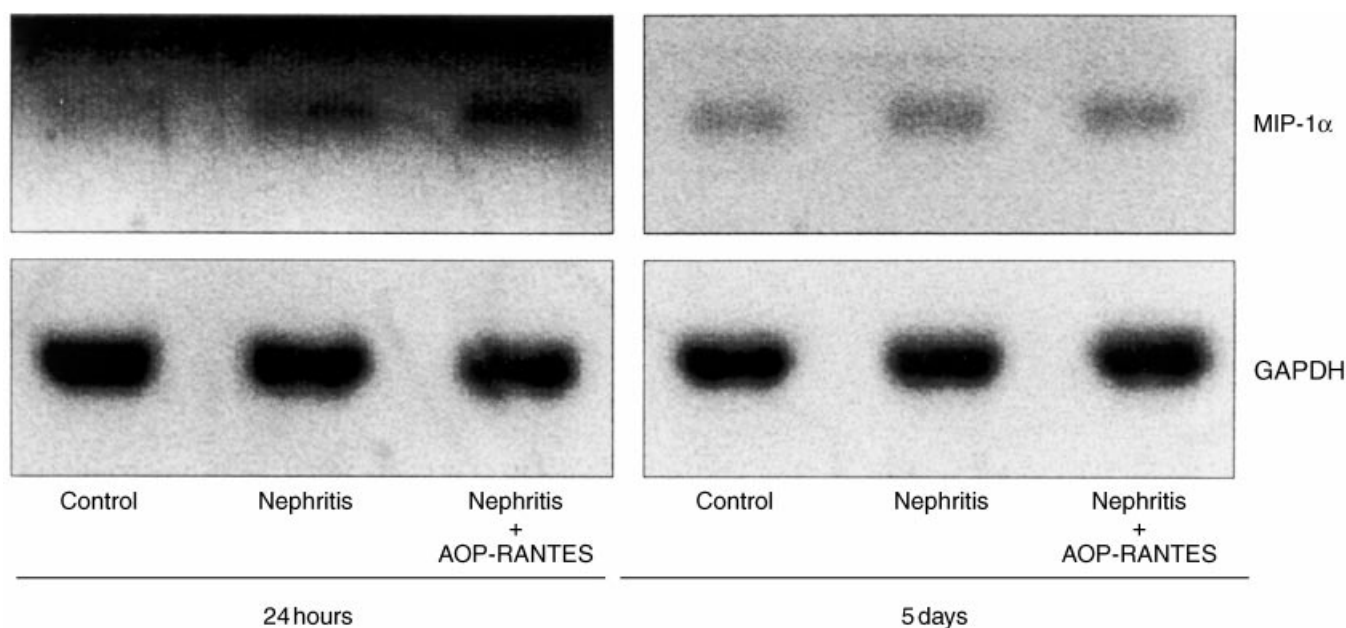


Fig. 6. Reverse transcription-polymerase chain reaction (RT-PCR) of macrophage inflammatory protein-1α (MIP-1α). RT-PCR demonstrates that MIP-1α RNA expression increases 24 hours after nephritis induction compared to control. Nephritic animals treated with AOP-RANTES showed similar level of MIP-1α expression. Five days after nephritis induction, there were no differences in the MCP-1α expression compared with the control.

Table 1. Densitometric analysis of the RT-PCR and Northern blot experiments

	Control	ATS	ATS + AOP-RANTES	Control	ATS	ATS + AOP-RANTES
	24 hr			5 days		
Northern blot experiments for RANTES (Fig. 5)						
RANTES/18 S	0.76	0.89	0.93	0.87	1.41	1.14
% of control	100	117	122	100	162	131
Northern blot experiments for MCP-1 (Fig. 5)						
MCP-1/18 S	0.24	2.1	1.7	0.47	1.12	1.14
% of control	100	875	725	100	238	242
RT-PCR experiments for MIP-1 α (Fig. 6)						
MIP-1 α /GAPDH	0.043	0.136	0.196	0.167	0.223	0.219
% of control	100	316	455	100	133	131

Exposed films were quantitated by using phosphoimager. Experimental analyses were independently performed for three different complete sets of experiments with qualitatively similar results.

To compare two distinct treatment groups (that is, nephritic vs. control or nephritic vs. treated animals), we applied the Mann–Whitney test. If not indicated otherwise, the results of the Mann–Whitney test always corresponded to a comparison of the nephritic versus treated animal.

RESULTS

White blood cell count

Application of ATS induced a significant fall in the white blood cell count from 10,900/ μ l to 5,030/ μ l cells after three hours ($P = 0.02$), which remained low up to 24 hours. AOP-RANTES did not significantly influence total white blood cells. Differential white blood cell count revealed that the reduction in total white blood cells was due to the fall of lymphocytes. AOP-RANTES did not significantly change the differential cell count of granulocytes and lymphocytes, but increased monocytes at three hours (ATS 240/ μ l vs. ATS + AOP-RANTES 960/ μ l, $P = 0.02$) and not significantly increased them at 24 hours (ATS 790/ μ l vs. ATS + AOP-RANTES 1280/ μ l, $P = 0.08$, $N = 4$ animals for each group).

Glomerular monocyte/macrophage infiltration

The induction of the glomerulonephritis resulted in a significant increase of glomerular monocyte infiltration from 0.33 ± 0.14 cells/glomerula cross-section (c/gcs) in controls to 2.88 ± 0.47 cells in nephritic rats at 24 hours (Figs. 1 and 2 A, B). Treatment of nephritic animals with AOP-RANTES prevented glomerular monocyte recruitment at 24 hours (nephritis + AOP-RANTES, 1.14 ± 0.08 c/gcs, $P < 0.02$; Fig. 2C). Five days after the induction of the diseases, glomerular monocyte/macrophage (M/M) infiltration in nephritic rats was still significantly higher than in controls (2.13 ± 0.22 cells). AOP-RANTES-treated animals had less glomerular M/M (1.4 ± 0.26) than nephritic animals; however, the difference did not reach statistical significance ($P < 0.08$).

Glomerular collagen type IV deposition

To study whether or not the AOP-RANTES treatment affected extracellular matrix formation in glomerulonephritis, the glomerular deposition of collagen type IV as a marker of extracellular matrix formation was determined. In ATS nephritis, collagen type IV formation is markedly enhanced at day 5, as has been demonstrated in earlier experiments [18]. The quantitative assessment revealed that AOP-RANTES reduced collagen deposition (from 2.09 ± 0.14 in nephritis to 1.45 ± 0.7 by almost 50% ($P < 0.02$; Figs. 3 and 4).

Chemokine mRNA expression

Glomerular mRNA expression of MCP-1 and MIP-1 α was hardly detectable in control animals, whereas there was a detectable basal expression of RANTES. Twenty-four hours following the induction of glomerulonephritis, mRNA expression of MCP-1 and MIP-1 α increased markedly, whereas RANTES was unchanged. At five days, MCP-1 and MIP-1 α mRNA levels fell, whereas RANTES expression increased. The treatment of nephritic rats with AOP-RANTES did not substantially affect the expression of RANTES, MIP-1 α , and MCP-1 mRNA at 24 hours and 5 days (Figs. 5 and 6 and Table 1).

RANTES and macrophage inflammatory protein-1 expression

Western blot analysis revealed an increased glomerular RANTES and MCP-1 protein expression after nephritis induction at 24 hours and 5 days. Treatment of nephritic animals with AOP-RANTES does not substantially alter the expression of RANTES and MCP-1 at 24 hours and 5 days (Fig. 7). Immunohistochemistry of MCP-1 showed a small amount of basal glomerular MCP-1 protein expression and revealed an up-regulated protein expression in nephritic animals (24 hr) and a similar level of expression in nephritic animals treated with AOP-RANTES (Fig. 8).

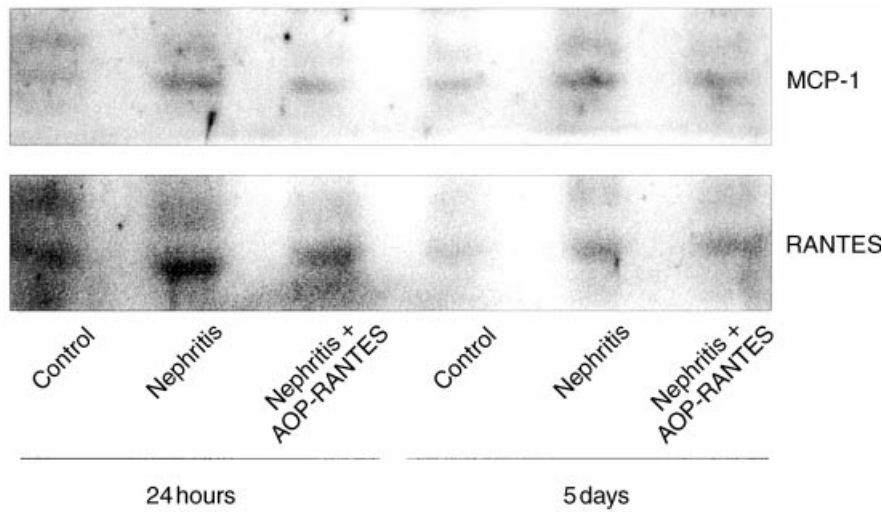


Fig. 7. Western blot analysis of RANTES and monocyte chemoattractant protein-1 (MCP-1). By Western blotting, glomerular RANTES and MCP-1 were detectable as a band at the size of 10 kDa, which was corresponding to the size of recombinant RANTES and MCP-1 protein (data not shown). In nephritic rats, the amounts of RANTES and MCP-1 were higher compared with controls. Treatment of nephritic animals with AOP-RANTES revealed a similar level of glomerular RANTES and MCP-1 protein expression at both time points.

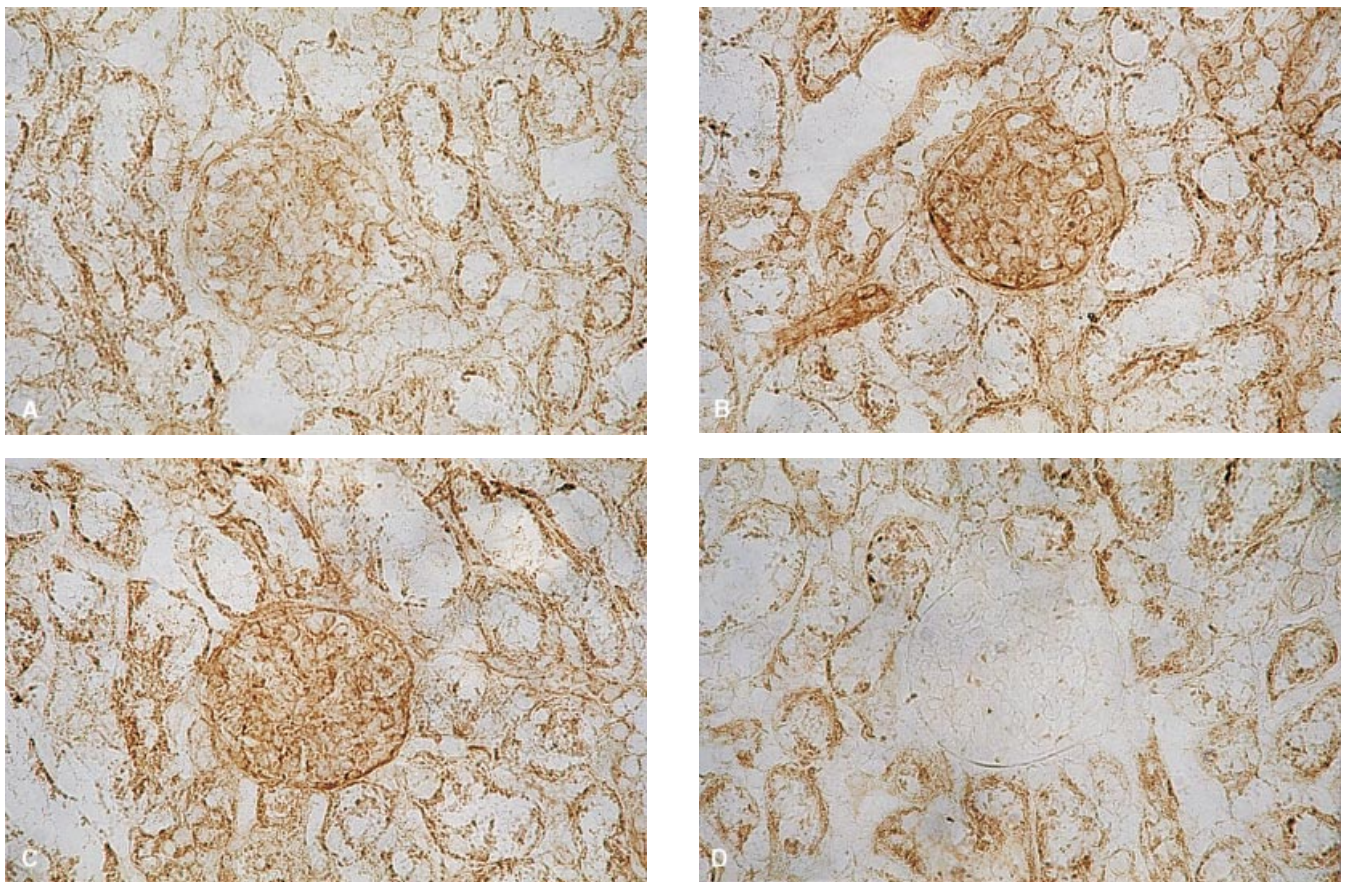


Fig. 8. Immunohistologic staining of glomerular monocyte chemoattractant protein-1 (MCP-1) expression. Immunohistologic staining of glomerular MCP-1 protein expression with an anti-MCP-1 antibody revealed a marked increased of glomerular MCP-1 24 hours after the induction of ATS nephritis (B) in comparison to the control group (A). Treatment of nephritic animals with AOP-RANTES revealed a similar level of glomerular MCP-1 protein. Staining with a negative control primary antibody (normal goat IgG) revealed no glomerular signal (D; $\times 400$).

DISCUSSION

The major finding of this study is that the chemokine derivative, AOP-RANTES, a novel CCR-5 chemokine receptor antagonist, prevents the glomerular infiltration of monocytes in experimental glomerulonephritis by over 60%.

Amino-oxypentane RANTES is a modified chemokine that binds on monocytes with a high affinity to the CCR-5 chemokine receptor [12, 13]. CCR-5 is the natural receptor of MIP-1 α , MIP-1 β , MCP-2, and RANTES, which induce chemotaxis in monocytes [13, 19]. Our data suggest that AOP-RANTES prevented glomerular monocyte infiltration by blocking the CCR-5 receptor, thereby inhibiting monocyte chemotaxis. Indirect evidence for such a mechanism derives from the finding that AOP-RANTES increased monocytes in the blood by more than 100% three hours after the induction of the disease, whereas the appearance in the injured tissue was reduced. This suggests that the blockade of CCR-5 prevented monocytes from migrating into inflamed tissues.

The evaluation of glomerular mRNA chemokine expression revealed that 24 hours after induction of the nephritis, MIP-1 α as a ligand of CCR-5 was up-regulated, whereas the expression of RANTES was quantitatively unchanged compared with controls. At five days, MIP-1 α had returned to almost control values, whereas RANTES expression was now up-regulated. The protein expression analysis of RANTES and MCP-1 showed similar results, despite an up-regulation of RANTES 24 hours after nephritis induction. This suggests that MIP-1 α was the major responsible mediator at 24 hours, whereas RANTES might play the major role in M/M recruitment at five days. Our data also suggest that the role of RANTES is less prominent because the reduction of M/M infiltration at day 5 was only moderate. The effect of AOP-RANTES on monocyte infiltration was incomplete, suggesting that other chemokines, apart from those that bind to the CCR-5, are relevant. In fact, in the same animal model we could demonstrate that the neutralization of MCP-1 reduces monocyte infiltration by approximately 40% [7]. Interestingly, treatment with AOP-RANTES did not decrease the RANTES, MIP-1 α , and MCP-1 mRNA or protein expression in glomeruli, which might be expected because infiltrating cells are known to be a major source of glomerular chemokine production. One might speculate that the blockade of the CCR-5 receptor may lead to increased levels of chemokines, such as MIP-1 α or MIP-1 β , which may induce other chemokines. The incomplete effect of AOP-RANTES adds more evidence that inflammatory cell recruitment cannot be prevented by a single receptor blocker or neutralizing antibody, but that combined approaches will be necessary.

By using a different approach, our data reconfirm that the depletion of inflammatory cells at an early time point

during the development of glomerulonephritis also improves the disease progression. Animals treated with AOP-RANTES had a moderate reduction of extracellular matrix deposition. It remains unclear which factors mediated this effect; however, we recently found that MCP-1 stimulates transforming growth factor- β expression in this glomerulonephritis and that anti-MCP-1 antibody reduces glomerular collagen formation [18]. It seems possible that other chemokines have similar effects. Lloyd et al showed that the RANTES antagonist Met-RANTES reduced the glomerular infiltration of inflammatory cells in an anti-glomerular basement membrane glomerulonephritis model but do not alter the glomerular extracellular matrix deposition [19]. These differences may be explained through the different animal models and the higher effectivity of AOP-RANTES to down-regulate the cell surface CCR-5 [13]. In summary, these data show that the CCR-5 receptor antagonist AOP-RANTES prevents the glomerular infiltration of monocytes in experimental glomerulonephritis. This approach may offer new possibilities to treat autoimmune injuries with chemokine receptor-blocking approaches.

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